antibodies **18**, the Fab' fragments, are immobilized through their sulfhydryl groups **21** to glass **25** via a derivatized silane linkage **30** (FIG. **1**). The sulfhydryl group is used for covalent attachment to the silane **35** via the bifunctional linking agent, succinimidyl-4-(p-maleimidophenyl)-butyrate **29**. The resulting orientation of the fragment with respect to the glass bead solid phase preserves the reactivity of the antigen-binding site. The advantage to this approach is three-fold. First, almost 100% of oriented immobilized antibody is active, in contrast to the 10 or 20% activity seen after non-covalent immobilization. This conserves expensive antibody not only in the initial solid phase preparation step, but also produces a reactive surface which is reusable. Second, the number of active antibody molecules which are covalently immobilized can be determined, allowing quantitative prediction of column activity. The third advantage derives from the fact that the preparation of non-covalently derivatized solid phases by nonspecific adsorption of antibody is frequently the most time-consuming step in ELISA-type assays. The ability to prepare stable, covalently immobilized antibodies which can be stored and reused greatly improves the time required to perform an assay.

Referring to FIG. **1**, the immunoreactor is prepared by immobilizing, on the column packing material, the antigen-binding portion of antibodies, the Fab' fragments **20**, through their sulfhydryl groups **21** to glass **25** via a silane linkage **30**. The silane reagent **35** depicted is 4-amino-butyldimethyl ethoxysilane, although any silane which can be derivatized to a primary amine group can be used. Under reflux, the silane reagent **35** binds to the glass beads **25**. The bifunctional linking agent **29** is then added and binds to the primary amine of the silane **35**. The completed silane linkage **30** immobilizes the Fab' fragments **20** through their sulfhydryl groups **21**. As a result, the covalently bonded complete antigen-binding site linkage **40** provides almost 100% activity of the immobilized antibodies. Alternatively, polystyrene is derivatized with amino groups by successive oxidation and reduction reactions. The modified polystyrene must be stable at pH 7 or 8 in aqueous conditions, and withstand treatment with various chaotropic agents, such as alcohols or glycine-HCl at pH 2, that are required for regeneration of analyte binding sites. Alternatively, intact molecules can be immobilized onto silicious or polystyrene surfaces using other chemistries.

As shown in FIG. **2**, when an analytical sample containing the compound of interest is injected into the FIIA system, interactions between analyte antigens **5** and the immobilized antibody fragments **20** occur on the surface of the beads **15** in the immunoreactor column. Detection of this interaction is mediated through the use of sensitized liposomes.

Liposomes **10***a* can compete for binding to immobilized antibodies **20** in the immunoreactor column with analyte molecules **5** in a sample; then for every liposome **10***a* which does not bind to the column **15** due to the presence of an analyte molecule **5**, approximately 1×10^5 marker (e.g., fluorescent) molecules are available for detection. Phospholipid molecules derivatized with antigen, antibody, Fab' fragments or class-specific binding agent are inserted into the membrane of each liposome. Alternatively, liposomes can be derivatized with virtually any molecule, hereforth referred to as a binding agent, in the following manner: phospholipid molecules derivatized with the vitamin biotin are inserted into the liposome membranes. The protein avidin, which has several binding sites for biotin, is added, and binds strongly to the biotin molecules on the liposomes. Then, a binding agent which is derivatized with biotin is added, and binds to avidin on the liposomes. These sensitized liposomes, which display binding agents on their surface, can now bind to antibody fragments on the solid support of the immunoreactor column or to other binding agents.

Liposomes can be noncovalently derivatized on their surfaces with virtually any analytical reagent. The interaction of biotinylated antigens or antibodies with liposomes containing biotinylphosphatidylethanolamine in the presence of avidin is so strong that it is as effective as a covalent bond.

In the competitive binding scheme shown in FIG. **2**, a sample and liposome reagent are injected into the system (step A). At the reactor, competitive binding occurs (step B). The number of liposomes which do not bind to antibody on the reactor are carried downstream (step C) where they can be detected, or, if they contain quenched fluorophore or electroactive molecules, they can enter a post-column mixing chamber. Here, surfactant is added (step D) which disrupts the membrane of liposomes, releasing their aqueous fluorescent or electroactive contents which pass into the detector (step E). Thus, for every liposome excluded from the column due to the binding of an analyte molecule, approximately 10^5 detectable molecules are released. The reactor is regenerated (step F) by disrupting the liposomes, and subsequently the interaction of binding agent or analyte and immobilized antibody, and a new sample can be injected (step A). In addition, the quantity of liposome marker molecules released in step F can also be subsequently determined (scheme II of FIG. **4**).

Different methods for the detection and quantification of marker compounds allow diverse applications of immunoassay methods. When using liposomes containing enzymes as marker compounds in their interior phase, disruption of the liposomes can allow the released enzymes to form enzyme reaction products which are more detectable than the enzymes themselves as marker compounds. This provides a further amplification of the detectability of the marker compounds. Color dyes may also be used as detectable marker compounds and in some cases do not require rupturing of the liposomes for detection and measuring.

The detectability of fluorophores in fluorescent marker compounds can also be accomplished by various methods. In one embodiment of the present invention, unquenched fluorophore marker compounds in the interior aqueous phase of sensitized liposomes can be detected with or without disrupting the liposomes. In another embodiment, quenched fluorophores from the interior aqueous phase of sensitized liposomes are detected after disruption of the liposomes results in unquenching of those fluorophores. Quenching characteristics of various fluorophores can be controlled by concentration gradients as well as other physical and chemical properties of the system.

A schematic of one prototype FIIA system is shown in FIG. **3**. The system operates under low-pressure and low flow-rates, and is simple and inexpensive to build. Microprocessor-controlled solenoid pinch valves X and tubing are rated to pressures of up to 2.1 bar. A sample solution S is aspirated into a sample injection loop SL using a vacuum V/W with a negative pressure of 0.03 bar. The vacuum may lead to a waste disposal at a system outlet. Because the sequence and timing of events